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PRINCIPAL INVESTIGATOR: Alexander G. Pantschenko, Ph.D.
Donald L. Kreutzer, Ph.D.

CONTRACTING ORGANIZATION: University of Connecticut Health Center
Farmington, Connecticut 06030-5335

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6. AUTHOR(S)
Alexander G. Pantschenko, Ph.D.
Donald L. Kreutzer, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Connecticut Health Center
Farmington, Connecticut 06030-5335

E-Mail: alexgp@nso2.uchc.edu

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13. ABSTRACT ***(Maximum 200 Words)***
Immunohistochemical (IHC) analysis of CXC cytokines (GRO and IL-8) their receptor, CXC-R2, in human breast cancer (HBC) patient samples; 2) IHC analysis of the IL-1 family of cytokines and receptors (potent inducer/regulator of CXC cytokines) in HBC patient samples; 3) Quantitation and correlation of the CXC and IL-1 families in HBC tissue homogenates with estrogen/progesterone receptor expression; 4) Evaluated *in vitro* bio activity of the CXC and IL-1 families; 5) Developed retrovector transfected HBC cell strains for *in vivo* studies; and 6) Developed a xenograft *ex ova* chick embryo model to evaluated the role of CXC and IL-1 in HBC tumor growth *in vivo*. **Conclusions:** 1) The CXC and IL-1 families of cytokines and receptors are present within the HBC tumor microenvironment; 2) There exists significant correlations between the expression of CXC/IL-1 families, estrogen receptor status and disease severity; 3) *In vitro*, IL-1 is a potent inducer of CXC cytokines; 4) CXC receptors are expressed not only in HBC patient tissue, but also on HBC tumor cell lines 5) CXC cytokines do not appear to be universally mitogenic in HBC cell lines, but more likely regulate activities (proliferation, migration) on specific HBC tumor subpopulations; and 6) A novel chick *ex ova* xenograft model can be used to evaluate HBC tumor growth and invasiveness.

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INTRODUCTION

Background. It is now well recognized that tumor growth (*i.e.* proliferation) and angiogenesis (*i.e.* the formation of new blood vessels) are critical to tumor growth and metastasis, and therefore important in cancer disease progression. One of the key mechanisms for regulating tumor cell proliferation and tumor angiogenesis is by networks of chemical signals that interact with specific receptors on cells. These chemical signals are referred to as cytokines. Cytokines and cytokine receptors can be loosely grouped based on their functional activity (*i.e.* mitogenic cytokines or growth factors induce proliferation and angiogenesis factors modulated new blood vessel formation). The cytokine Growth-Regulated Oncogene (GRO) protein is a member of the CXC family of cytokines (based on conserved cystine residues) of related cytokines which are best know for their ability to induce a migrational response for various cell types. These chemotactic cytokines (or chemokines) are now known to also be involved in the modulation/induction of proliferation and angiogesesis (see below). Members of this family include; GROα/MGSA, GROβ, GROγ, IL8, NAP-2, ENA-78 and granulocyte chemoattractant protein-2. The receptors for these chemokines were originally described as IL8-R1 and IL8-R2, but are newly designated CXC-R1 and CXC-R2. IL-8 binds to both receptors with high affinity, whereas GROα /MGSA, GROβ, GROγ, and NAP-2 bind with high affinity to IL8-R2/CXC-R2 (K_d 0.2-2.5nM) and low affinity to IL8-R1/CXC-R1 (K_d 200-500nM), (1-5). Therefore, regulation of tumor cell proliferation and angiogenesis occurs *via* the expression and subsequent receptor interaction with cytokines. While very little has been described regarding the role of CXC cytokines in human breast cancer, there is evidence suggesting that this family of cytokines play an important role in other tumor systems.

CXC Cytokines (GRO and IL-8) and Receptors in Cancer: Recently, the CXC cytokine family members, Growth-Regulated Oncogene/Melanoma Growth Stimulatory-Activity – Protein (GRO/MGSA), and Interleukin 8 (IL-8) have been demonstrated in other tumor systems to be: ***a)*** an autocrine tumor cell growth factor for malignant melanoma, liver and pancreatic cancer and ovarian cancer (6-11), ***b)*** an in vitro (macrophage derived) and in vivo (the rat cornea model) angiogenesis factor (12-14), ***c)*** a potent *in vitro* migration factor for neutrophils and breast cancer cells (15-17), and ***d)*** able to support and augment melanoma and ovarian cancer tumor formation in the xenograft nude mouse model (11, 18-21). Additionally IL8 is; ***a)*** predictive indicator of therapeutic response and prognosis of patients with recurrent breast cancer (22), ***b)*** shown by ourselves, that IL8 is expressed by tumor cells and the receptors are expressed on both tumor cells and vascular endothelial cells from breast and neuroblastoma patient tissue samples (23-24). Furthermore, IL8 expression is inversely correlated with ER/PR expression in human breast cancer, suggesting IL8 expression is associated with poor prognosis (25), ***c)*** it has been shown that inhibition of the common GRO/IL8 receptor, CXC-R2 by antisense oligonucleotides inhibits tumor cell proliferation in vitro and in the xenograft nude mouse model (26).

This data suggests that tumor cell derived (TCD) CXC cytokines (GRO and/or IL8) may play a significant role in human breast cancer tumor growth and therefore understanding the role of these cytokines will potentially lead to exciting new therapeutic approaches in the fight against breast cancer.

***Hypothesis*:** Based on our own preliminary data and a recent literature review, we hypothesize that in human breast cancer (HBC), the local expression of GRO and the GRO-Receptor (CXC-R2) by tumor cells supports tumor growth and metastasis by promoting tumor proliferation directly (as an autocrine growth factor), but also augments tumor growth indirectly by supporting tumor angiogenesis *via* expression of CXC-R2 on vascular endothelial cells (as a paracrine angiogenic factor). The duel activation of these essential protumorgenic pathways by CXC cytokines and the interaction/cooperativity between subpopulations expressing these cytokines and receptors is critical for successful tumor growth and metastasis.

BODY OF REPORT.

(ALL FIGURES FOR THIS SECTION CAN BE FOUND IN APPENDIX).

Overview.

In our original human breast cancer (HBC) study proposal, we hypothesize that the local expression of GRO and GRO-Receptor (CXC-R2) by tumor cells supports tumor growth and metastasis by promoting tumor proliferation directly (as an autocrine growth factor), but also augments tumor growth indirectly by supporting tumor blood vessel development (as a paracrine angiogenesis factor). In support of this hypothesis we defined the following specific aims: *1)* characterize GRO expression and correlate CXC-R2 expression of human breast cancer tumor tissue with major diagnostic and prognostic indicators, *2)* characterize human breast cancer tumor cell activation (*i.e.*, GRO expression and proliferation) using *in vitro* models, and *3)* determine the role of GRO/CXC-R2 expression in tumor growth and angiogenesis in a xenograft model of human breast cancer. Our studies have supported the hypothesis that GRO plays a role in HBC progression, but more importantly, our studies also suggest cytokine/cytokine receptor mediated cell cooperativity among tumor and normal cell subpopulations are important in driving the processes of HBC progression. We have observed that Interleukin-8 (IL-8, related to GRO in the CXC cytokine family) expression parallels GRO expression. Furthermore, the CXC-Ls (GRO and IL-8) can be controlled by the IL-1 family of cytokines and receptors. Therefore, we have extended our hypothesis (and study) to include IL-1 activation/modulation of the CXC-L (Ligands) and CXC-R (Receptors) in HBC progression. Our working hypothesis (Figure 1) is that within the breast tumor microenvironment, functional tumor cell subpopulations can be distinguished by their expression of IL-1 (IL-1α and IL-β) and CXC cytokines (IL-8 and GRO) as well as their related Receptors, *i.e.* (IL-1Rs: IL-1RI and IL-1RII) and (CXCR: CXC-R1 and CXC-R2). We further hypothesize that these subpopulations of HBC tumor cells work synergistically to promote tumor progression by inducing angiogenesis and tumor cell activation (*e.g.* proliferation, migration, and invasion) *via* IL-1 and CXC Pathways. Thus, the network of IL-1 and CXC cytokines and receptors, present on the various HBC subpopulations, work in a coordinated fashion to control tumor cell function, and therby tumor progression *in vivo*. Provided below is a brief summary of the data obtained in support of our hypothesis in specific sections.

SUBSECTION 1: Levels of CXC-Ligands (GRO and IL-8) and CXC Receptors (CXC-R1 and CXC-R2) in Human Breast Cancer (HBC) Patient Tissue.

Study 1: GRO and IL-8 Levels in HBC Tumor Homogenates.

Key data in support of our hypothesis on the role of IL-8 and GRO in HBC is demonstrated by both the presence and relationships of the CXC cytokine family in HBC patient tissue. We have already described that IL-8 expression is associated with negative ER and PR status in human breast cancer homogenates, suggesting IL-8 expression is a marker of poor diseases outcome (see Reference 25). Extending these observations, we used human breast cancer homogenates and ELISA technology to demonstrate that: *1)* GRO and IL-8 are present in human breast cancer tumor tissue; *2)* IL-8 levels in HBC tumor tissue are significantly higher than GRO levels; *3)* known inducers of GRO and IL-8 (IL-1 and TNF family of cytokines and receptors) are present in HBC tumor tissue, and *4)* The prognostic indicators ER and PR are detectable in these samples (Figure 2). Although detecting these cytokines in HBC tissue is significant, clearly determining whether these cytokines, cytokine receptors, or prognostic factors, correlate in any significant way is critical. Thus we next determined the correlations between the various factors in the HBC tissue.

SUBSECTION 2: Levels of IL-1 agonists (IL-1α and IL-1β), IL-1 antagonists (IL-1ra), and IL-1 Receptors (IL-1 RI and IL-1 RII) in Human Breast Cancer (HBC) Patient Tissue.

Study 2: IL1-α, IL-β, and IL-1ra Levels in HBC Homogenates.

To extend our studies on the role of the IL-1 family of cytokines and receptors in HBC, we next determined the levels of the IL-1 cytokine in HBC tissue homogenates (Figure 2). Analysis of breast tumor homogenates indicated that 71 of 82 (87%) cancer specimens had detectable levels of IL-1α (*i.e.* >5 pg/ml). The levels of IL-1α ranged from 10.0 pg/mg total protein (TP) to 25.5 pg/mg TP, with a mean for

the 82 samples of 17.6 ± 3.9 pg/mg TP. Analysis of tumor homogenates for IL-1β antigen indicated that 96 of 101 (95%) cancer specimens had detectable levels of IL-1β (i.e., > 5 pg./ml). The IL-1β levels ranged from 9.2 pg./mg TP to 14.9 pg./mg TP. The mean IL-1β value for the 101 samples was 12.02 ± 1.4 pg./mg TP. ELISA analysis of the HBC homogenates demonstrated the presence of IL-1α and IL-1β in HBC tumor homogenates in approximately equal concentrations (17.6 ± 3.9 pg./mg TP and 12.0 ± 1.4 pg./mg TP) respectively. These data clearly demonstrate the presence of significant levels of IL-1α and IL-1β in HBC tissue (see Reference 27). The IL-1 antagonist, IL-1ra was also detected in the HBC tissue homogenates and correlated with ER status of the patients (see Reference 28). This observation suggests that low concentrations of the IL-1 antagonist, IL-1ra, are associated with indicators of poor disease outcome (ER negative status). In this current study, we evaluate and correlate expression of the IL-1 family of cytokines and receptors with expression of GRO. One interpretation of this data is that the lack of IL-1 antagonist in the tumor microenvironment (TME), likely allowed IL-1 mediated HBC tumor activation, productive of pro-tumorogenic factors such as IL-8 and GRO. These data clearly implicate the IL-1 family of cytokines in HBC tumor activation and progression. Thus it therefore becomes critical to determine the distribution of IL-1receptors within the tumor microenvironment, particularly IL-1R expression on HBC cells.

Study 3: IL-1 Receptor Levels in Human Breast Cancer Homogenates.

Since we have demonstrated expression of IL-1 agonist and antagonist in HBC tissue homogenates we next determined the levels of IL-1R in these tumor tissues. We determined IL-1RI and IL-1RII levels in HBC tumor homogenates using standard ELISA technology, and correlate this data with results for IL-1 cytokine expression in these same samples (Figure 2 and Subsection 3 below). Quantitative IL-1R studies of the tumor homogenates demonstrated not only detectable levels of IL-1RI (15.37 +/- 4.18 pg/mg total protein) and IL-1RII (58.34 +/- 8.25) in the HBC tumor tissue, but that IL-1α IL-1β, IL-1ra, IL-8 and GRO were also present. These results support our hypothesis on the role of IL-1 and IL-1R in tumor cell activation and disease progression. Since IL-1α and IL-1β are known to induce expression of the GRO and IL-8, our homogenate studies raise the question of the relationship and prognostic value of the IL-1/CXC levels.

SUBSECTION 3: Correlation of GRO and IL-8 Levels with Prognostic Factors and Pro-Tumorigenic Cytokines and Receptors in Human Breast Cancer (HBC) Patient Tissue.

Study 4: GRO and IL-8 Correlations in Human Breast Tumor Homogenates.

We next correlated the IL-8 and GRO cytokine levels in HBC homogenates with the results for the IL-1 family of cytokines and receptors in the matched homogenates (Figure 2). The results of these studies (Figure 3) demonstrated that: *1)* IL-8 expression is inversely related to estrogen receptor (ER) expression (*i.e.* high IL-8 levels correlated with low ER expression); *2)* GRO expression is inversely related to estrogen receptor (ER) expression (*i.e.* high GRO levels correlated with low ER expression); *3)* IL-8 and GRO levels correlated with a number of the IL-1 family of cytokines; *4)* GRO expression follows IL-8 expression (Figure 4) and *5)* GRO and IL-8 levels are significantly different between ER+ and ER- HBC tissue homogenates (Figure 5). Since low/no ER expression is associated with an unfavorable disease outcome, these data suggest that high GRO and IL-8 expression is associated with a poor prognosis. Thus, our data demonstrates that both IL-8 and GRO correlate with the important prognostic indicators ER and/or PR, and is critical in supporting our hypothesis that both IL-8 and GRO are important cytokines in HBC growth and progression. Additionally, our data demonstrates that IL-8 and GRO correlate with members of the IL-1 family of cytokines, suggesting that IL-1 may be a major inducer of IL-8 and GRO *in vivo* as well as *in vitro* (see Subsection 6 below). Although these data clearly support the hypothesis that HBC tissue express IL-8 and GRO, it is important to determine the cellular sources of these cytokines and determine what cells are the potential effector targets of these cytokine (*i.e.* receptor expression). Therefore, we next examined the distribution of the CXC and IL-1 family of cytokines and receptors in HBC patient tissue.

SUBSECTION 4: Immunohistochemical Analysis to Determine the Distribution of the CXC Family of Cytokines and Receptors in HBC Patient Tissue.

Study 5: GRO and IL-8 Distribution in Human Breast Cancer Tissue.

Since a wide variety of cells including leukocytes and tissue cells are known to express GRO and IL-8, it is critical to determine the potential sources of GRO and IL-8 in HBC tissue. Therefore, we utilized standard IHC techniques and antibodies specific for human GRO and IL-8 to investigate the distribution of these cytokines in human breast tissue. These studies demonstrated that: *1)* GRO was expressed by invasive (INV) malignant breast cancer cells, DCIS and normal (NOR) mammary epithelial cells (Figure 6); *2)* IL-8 is expressed by invasive (INV) and ductal carcinoma *in situ* (DCIS) breast cancer cells *in vivo* (Figure 7); and *3)* ductal epithelial cells in benign (BEN) breast tissue express lower levels of IL-8. Our results demonstrate that GRO expression is associated with HBC tumor as well as normal mammary epithelial cells. Similarly, IL-8 is also expressed in tumor cells and normal epithelial cells in the mammary gland. However, while GRO is only found in tumor/epithelial cells, IL-8 is also expressed on vascular endothelial cells (VEC). This expression of IL-8 is more often associated with small and large vessel endothelial cells (SVEC and LVEC) in malignant samples, but not observed in LVECs in benign samples. Additionally, to support our hypothesis that GRO and IL-8 can act synergistically in both an autocrine and paracrine fashion, we must demonstrate the presence of CXC receptors on the surface of the HBC tumor cells.

Study 6: GRO and IL-8 Receptors (CXC-R1 and CXC-R2) Distribution in Human Breast Cancer Tissue.

CXC-R2 is a common receptor for both GRO and IL-8, while CXC-R1 only binds IL-8. We have previously demonstrated CXC-R1 and CXC-R2 expression in HBC (see Reference 23) and associated it's expression with IL-8. In this study, CXC-R2 is (Figure 8) correlated with GRO expression and a summary of the immunohistochemical results describing the expression of IL-8, GRO, CXC-R1 and CXC-R2 are presented in Figure 9. IL-8 and it's receptors (CXC-R1 and CXC-R2) can be found on not only tumor cells, but also on vascular endothelial cells. Similarly the GRO receptor (CXC-R2) is expressed on tumor cells and vascular endothelial cells and importantly, CXC-R2 appears to be up-regulated or preferentially expressed in malignant breast tumor tissue. However, unlike IL-8 expression, which can be observed on both tumor and vascular endothelial cells, GRO can only be found on tumor and breast epithelial cells. These IHC studies clearly support the hypothesis that HBC cells are the major source of GRO and IL-8 in HBC tissue. However, to truly demonstrate that HBC cells can synthesize IL-8 and GRO, specific *in vitro* studies using isolated HBC tumor cells are required, *i.e.* cell lines (see Subsection 6 below).

SUBSECTION 5: Immunohistochemical Analysis to Determine the Distribution of the IL-1 Family of Cytokines and Receptors in HBC Patient Tissue.

Study 7: IL-1α, IL-1β, and IL-1ra Distribution in HBC Tissue.

We have previously demonstrated (see Reference 27 & 28) the distribution of IL-1 agonists (IL-1α, IL-1β) and antagonists (IL-1ra) in HBC tumor tissue using standard IHC techniques. IL-1α was consistently expressed in the tumor cells of all of the specimens from patients with both invasive (32/32) and ductal carcinoma *in situ* (DCIS), (10/10). IHC analysis of the HBC specimens for IL-1□ indicated that tumor cells showed a consistent cytoplasmic staining pattern for IL-1β invasive 88%, (23/26) DCIS 78% (7/9). Analysis of normal ducts found in the specimens indicated that ductal epithelium in 77% (20/26) of the invasive HBC, but only 56% (5/9) of the DCIS HBC samples showed positive staining for IL-1β. Finally, All of the malignant specimens expressed IL-1ra, 100% [invasive (23/23) and DCIS (5/5)]. A number of malignant specimens contained areas of normal or hyperplastic ductal morphology adjacent to the tumor. A majority of the adjacent ductal epithelial cells of the invasive [86%(20/23)] and DCIS [75%(4/5)] HBC specimens expressed IL-1ra. Analysis of the intensity of staining for the tumor cells and ductal epithelial cells indicated that tumors cells consistently staining more intensely than ductal epithelial cells for all IL-1 cytokines. These IHC studies demonstrate that: 1) the IL-1 family of cytokines is present in the tumor microenvironment; 2) that tumor cells themselves are likely a major source of these cytokines within the tumor microenvironment and 3) that subpopulations of HBC tumor cells, based on IL-1 cytokine

expression (IHC), exist in HBC.

Study 8: IL-1 Receptor Distribution in Human Breast Cancer.

Clearly the data presented above demonstrates the presence of IL-1 cytokines (agonists and antagonists) in HBC tissue, but this data also raises the question of which cells within the tumor microenvironment express the IL-1 receptors. To begin to answer this question we conducted IHC analysis of the human breast disease (HBD) tissue for the IL-1 receptors, IL-1 RI and IL-1RII. HBC tumor cells consistency stained positive (87-100%) for both IL-1RI and IL-1RII, in both DCIS and invasive breast cancer (Figure 10). This data clearly demonstrates that the tumor cells express IL-1 receptors, thus supporting our hypothesis that IL-1 cytokines (agonists and antagonists) can regulate tumor cell activation within the tumor microenvironment.

Subsection 6: *In Vitro* Expression of IL-8 and GRO By Human Breast Cancer Cells.

Although these data clearly support the hypothesis that HBC cells express IL-8 and GRO, it is imperative to directly demonstrate that HBC tumor cells can express these cytokines *in vitro*. Using *in vitro* models of HBC will allow us the opportunity to determine whether IL-1 cytokines can directly induce IL-8 and/or GRO in HBC tumor cells. The results of the studies could also provide insights into HBC tumor cell subpopulations. Thus we undertook the following investigations. We hypothesized that HBC tumor cells can express CXC cytokines, and based on this expression, subpopulations of HBC cells can be demonistrated *in vitro* and *in vivo*. We further hypothesize that this CXC expression is under the control of IL-8/GRO inducing cytokines [*i.e.* Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNFα)]. To directly test this hypothesis, we: 1) determined the ability of breast cancer cells (BCCs) and breast epithelial cells (BECs) to produce IL-8 and GRO *in vitro,* and 2) determined the ability of IL-1 and TNF to regulate IL-8 and GRO expression by these cells.

Study 9: IL-8 and GRO Expression in Human Breast Cells *in vitro*.

Basal IL-8 expression, was determined in culture supernatants from normal human breast cells (HMEC), estrogen dependent human breast cancer cell lines (MCF-7, T-47 D, and ZR-75-1) and estrogen independent human breast cancer cell lines (BT-20 and MDA-MB-231). HMEC and MDA produced the highest basal expression of IL-8 and GRO, when compared to the other cell lines used in this study. As stated earlier, IL-1 cytokines are known to be potent inducers of IL-8 and GRO expression in leukocytes and normal tissue cells. Therefore, we extended our studies to investigate the ability of IL-1α and IL-1β to induce IL-8 and GRO expression in normal and malignant breast epithelial cells. Normal BECs showed marked increased expression of IL-8 and GRO, when treated with either IL-1α or IL-1β (Figure 11). This data demonstrates that IL-1 cytokines are potent inducer of IL-8 and GRO in non malignant BEC. We next evaluated the ability of IL-1α or IL-1β to induce IL-8 and GRO expression in HBC cell lines. These studies demonstrated (Figure 12) that: 1) IL-1 induced both IL-8 and GRO expression in the HBC cells, but a clear heterogeneity in IL-8 and GRO responses to IL-1 cytokines existed; 2) IL-8 expression was consistently higher that GRO expression in the responsive cell lines; 3) HBC cell lines that demonstrate IL-1 induced expression of IL-8 constantly also demonstrate increased GRO expression to IL-1 induction; 4) that IL-1 cytokines induced significant IL-8 and GRO expression in the estrogen independent HBC cell lines (BT-20 and MDA-MB-231); and 5) one of the three estrogen dependent lines (MCF-7) showed no increased IL-8 or GRO expression when stimulated with IL-1 cytokines, but interestingly the T-47-D cell line did not have an increased expression of IL-8, but did show a modest increase in GRO expression when stimulated with IL-1 cytokines. Since we have demonstrated the existence of IL-1, IL-1R and CXC cytokines in HBC cells and tissue, it is critical to demonstrate the existence of CXCR in HBC cells and tissue. To this end, we undertook investigations into CXC-R expression in HBC cells (see Study 10).

Subsection 7: *In Vitro* Expression of CXC-R2 Receptor By Human Breast Cancer Cells.

Study 10: IL-8/GRO CXC Receptor Expression in HBC Cell Lines and Human Breast Cancer Tissue Homogenates.

To demonstrate functional activity of the CXC cytokine/receptor network, we next examined

expression of GRO/IL-8 receptor CXC-R2 in HBC cell lines. In these studies we were able to demonstrate that CXC-R2 receptor is constitutive expression in MDA-MB-231 cell lines by Western blot (Figure 13). Interestingly, CXC-R2 expression was not modulated by the addition of IL-8, TNFα, LPS, PMA or dexamethasone to the cell cultures. In this same study, we also demonstrate CXC-R2 expression in HBC tumor homogenates. While this study represents only a single HBC cell line, it does demonstrate CXC-R2 *in vitro* expression in breast cancer cells and HBC tissue homogenates. We are currently evaluating other HBC cells for receptor expression.

Subsection 8: *In Vitro* Biological Activity of the CXC/CXC-R Pathway in HBC cells.

Study 11: Since we demonstrate CXC-R2 expression on HBC tumor cells and hypothesize that GRO acting through it's receptor can induce tumor cell proliferation, we undertook studies to investigate the ability of GRO and IL-8 to stimulate HBC growth *in vitro*. Our preliminary data (Figure 14) suggests that GRO and IL-8 do not have mitogenic activity for MDA-MB-231 cells under the culture conditions we tested. We are currently surveying other human breast epithelial and tumor cells for GRO and IL-8 mediated proliferation responsiveness. Additionally, we are interested is evaluating other biological activities (*e.g.* chemotaxis) that may influence tumor progression and which are mediated by the CXC/CXC-R pathway. While our HBC *in vitro* studies demonstrating CXC/CXC-R pathway activity in tumor progression is ongoing, we are addressing biologically activities (*i.e.* growth, invasiveness, and angiogenesis) in a novel xenograft model of HBC (see Subsection 9, below).

Subsection 9: Xenograft Model of Human Breast Cancer.

Study 12: The *Ex Ova* CAM Model of Human Breast Cancer (xenograft).

Previously we have utilized the human-murine xenograft model to investigate the roles of cytokines in cancer progression. Although this is a well-accepted model, it is extremely time, labor and cost intensive (Figure 15). As an alternative to the murine models, we have developed a chick embryo Chorioallantoic membrane (CAM) model, as an *in vivo* model to study tumor cell proliferation, migration, invasiveness and angiogenesis. The ability of human tumors to grow in chick embryo CAMs is do to the fact that the chick embryo, like the nude mouse, is immuno deficient, and thus can be used in xenograft studies. Previous studies have utilized the *in ova* chick chorioallantoic membrane, to test the presence of angiogenic factors as well as inhibitors of angiogenesis. In addition, some investigators have utilized the *in ova* CAM as a model for growing both tumor tissue, as well as tumor cells. These studies are limited by the difficulties of the *in ova* CAM model which are overcome by our recently developed *ex ova* CAM model (Figure 16). In the *ex ova* model, the entire fertilized egg content (at day 3) is placed in a petri dish after carefully cracking the shell. The resulting embryo and CAM are incubated at 37-38^{O}C for additional days. Although, our new model involves the total removal of the egg shell and replacing it with a petri dish, as such, it should be emphasized that this is a completely *in vivo* model. Our *ex ova* CAM model represents an elegant *in vivo* model which takes advantage of the extensive vasculature available on the chick embryo CAM.

Study 13: The Human Breast Cancer *Ex Ova* CAM Model.

For our HBC tumor cells, we have planted the tumor at day 9-12 gestation (when the CAM is well established) and followed tumor cell growth for an additional 5 days (see below). In this unique model, by transferring the entire fertilized egg content to a petri dish (*ex ova* model) it has the advantage of providing ready access to the embryo and it's highly vascularized membranes (Figure 16). Not only does this *ex ova* systems allow for easy introduction of various cells types, but also various test samples, such as factors that modulation tumor progression (*e.g.* cytokines, angiogenesis or anti- angiogenesis factors). Additionally, the *ex ova* system allows us to easily monitor, by direct microscopic observation, tumor cell morphogenesis, invasiveness, angiogenesis and growth on the chick CAM. In our preliminary studies we investigated the distribution of tumor cells on the CAM, using MDA-MB-231 breast cancer cells transfected with a vector expressing the green fluorescent protein (GFP) gene or red fluorescent protein (DsRed2), (Figure 17). We have utilized these cells as a simple method of tracking the presence and survival of cells in the CAM model (Figure 18). These studies demonstrate our ability to culture and monitor HBC cells grown on the CAM.

Study 14: The *Ex Ova* CAM Model to Study Human Breast Cancer Growth and Progression.

Although the gross morphology suggests tumor cells are present in the CAM at 48 hours post placement. To directly demonstrate the presence and growth of the tumor cells, we histologically examined MDA-MB-231 cells grown on the CAM. Control (no tumor cells) as well as CAMs with tumor cells, were fixed, processed, paraffin embedded, sectioned, and stained by H&E. As can be seen in Figure 19, these studies indicate the feasibility of using the chorioallantoic membrane as a simple model to look at both tumor cell growth and invasion. We feel that this model will also be extremely useful in surveys/screening of various cell lines, clones and gene transfer variants which are pertinent to our hypothesis in understanding the role of the IL-1/CXC families of cytokines and receptors in human breast cancer progression.

KEY RESEARCH ACCOMPLISHMENTS
(ALL FIGURES FOR THIS SECTION CAN BE FOUND IN APPENDIX).

- Based on our observation that IL-1 (relative to TNF, LPS, and Fibrin) is a potent *in vitro* inducer of GRO, (and the related cytokine IL-8), in subpopulations of human breast cancer (HBC) cells (Figure 11&12) we have expanded our *in vivo* and *in vitro* studies to focus on the IL-1 family of cytokines and receptors and investigate their role in GRO/IL-8 activation and up-regulation. Therefore, we have included IL-1 in our hypothesis on the role of CXC cytokines in HBC progression (Figure 1).

- Measured GRO levels as well as the levels of other pro-tumorigenic cytokines and receptors [IL-8, IL-1α, IL-1β, IL-1ra, IL-1RI, IL-1RII, Estrogen Receptor (ER), and Progesterone Receptor (PR)] in HBC tissue homogenates by ELISA (Figure 2).

- Correlated the GRO levels of expression (pg/mg of total protein) with the expression of other pro-tumorigenic cytokines and receptors (IL-8, IL-1α, IL-1β, IL-1ra, IL-1RI, IL-1RII, ER, and PR) in human breast cancer homogenates (Figure 3).

- Demonstrated that GRO expression in HBC tissue homogenates correlates with IL-8 expression and both GRO and IL-8 inversely correlates with the prognostic indicator ER (estrogen receptor), (Figure 4&5). This suggests that increased levels of GRO and IL-8 in HBC patient tissue is associated with poor disease outcome.

- Demonstrated breast tissue expression of GROα by human breast cancer (HBC) tumor cells (invasive and ductal carcinoma *in situ*) and normal breast epithelial cells (mammary reduction/fibrocystic), using immunohistochemistry (IHC), (Figure 6).

- We have decided <u>not</u> to stain HBC tissue with anti-VWF and anti-CD31 to quantitate microvessel density. (as described for year 01), since GROα is apparently equally expressed in malignant and benign breast tissue as observed by IHC, Therefore, GRO expression does not correlate with vessel count in HBC.

- Compared breast tissue expression of GROα with the expression of CXC-R2 (GRO receptor) and other pro-tumorigenic cytokines and receptors [IL-8, IL-1α, IL-1β, IL-1 Receptor Antagonist (IL-1ra), IL-1 Receptor I (IL-1RI), IL-1 Receptor II (IL-1RII)] by HBC tumor cells (invasive and ductal carcinoma *in situ*) and normal breast epithelial cells (mammary reduction/fibrocystic), using immunohistochemistry. (Figure 7-10).

- Demonstrated *in vitro* HBC subpopulations based on constitutive and inducible GRO and IL-8 expression (Figures 11&12).

- Demonstrate CXC-R2 expression in HBC cell lines and HBC tissue homogenates by Western blot analysis (Figure 13).

- Established *in vitro* proliferation assays of HBC cells and initially determined that GRO and IL-8 are not mitogenic for MDA-231 cells (Figure 14).

- In addition to our original study, we are evaluating *in vitro* activation of HBC cells by GRO/IL-8/IL-1 to include induction of chemotaxis and invasiveness.

- Developing a novel xenograft model (*ex ova* CAM model) to determine the role of GRO in HBC angiogenesis, invasiveness and metastasis (Figures 15,16,18 and 19).

- To support our xenograft model we have established retrovector transfected HBC cell strains and clones. These cells express either green fluorescent protein (GFP) or red fluorescent protein (DsRed), (Figure 17&18). Additionally, these cells are being cloned and evaluated for expression of GRO/IL-8 and CXC-R. Establishing this cell model combined with our xenograft models will likely allow us to gain novel insights into the role of the CXC cytokine and receptors in HBC progression.

KEY TRAINING ACCOMPLISHMENTS

1. ACADEMIC TRAINING ACCOMPLISHMENTS.

- Seminars, presentations, and lecture series. The University of Connecticut Health Center is a well recognized and active medical/basic research center with many opportunities for participation and interactions with scientists in various fields, including; immunology, cancer biology and cell biology. Attending these types of presentations is most helpful is maintaining a high level of knowledge required to successfully move forward research in human breast cancer and important in career development.
- Interaction with clinicians, faculty, and other scientists. The unique working environment at UConn is very supportive allowing for interaction with other members of the scientific community that are always willing to help. This is important for the discussion of scientific concepts, principals and techniques. As such, these assets make for a stronger ability to contribute to the area of breast cancer research.
- Research focus. My previous professional research experience and doctor dissertation was primarily related to immunology, normal mammary gland biology, and cell biology. This fellowship has allowed me to focus and gain knowledge in the field of human breast cancer.

2. TECHNICAL TRAINING ACCOMPLISHMENTS.

- Cancer biology and pathology. Research in human breast cancer has strengthened my background in the field of cancer biology. Similarly, a large focus of the study relies on the ability to distinguish between different types of human breast diseases and I have had to learn breast pathology and histopathology. Additionally, I have worked closely with pathologists and histotechnologists to learn clinical pathology and human tissue sample acquisition and preparation.
- Immunohistochemistry and cytokine biology. I have had to develop and use techniques to demonstrate cytokine presence in human breast cancer patient samples by IHC and techniques related to cytokine biology
- Molecular biology. Techniques utilizing molecular biology are a new addition to my list research tools that are being applied to my cancer studies. While we are still in the process of acquiring samples related to this study of human breast cancer using a molecular biology approach, I have been developing techniques to characterize cytokine induction and expression

using RT-PCR. Along the way I have learned isolation of RNA, primer design, PCR, DNA quantitation, and electrophoresis. Additionally, we are developing methods for transfection of cDNA for cytokines and marker proteins [green florescent protein (GFP)] which will be useful for our year 03 studies using the nude mouse xenograft model.

3. CAREER/ADMINISTRATIVE SKILL DEVELOPMENT

- Publications/Grant Writing. As part of my post-doctoral position, I have been involved with the writing of manuscripts and grants. These efforts have improved by communication skills and hopefully, some of the grant proposals will attract funding which leads to career development.
- Administrative. As a post-doc, I have also had to work with administrative issues related to doing research. This would include writing reports for the Animal Care and Usage Committee, Institutional Review Board for using human anatomical substances, the grants/research office for grant submissions and preparing budgets. All these will surely prepare me for a successfully career as an academic researcher.

REPORTABLE OUTCOMES

1. MANUSCRIPTS.

- **In Vitro Demonstration of Breast Cancer Tumor Cell Subpopulations Based on the Expression of Interleukin-8.**
  Alexander G. Pantschenko, Irina Pushkar, Lauri J. Miller, YanPing Wang, Kathleen Anderson, Ziv Peled, Scott H. Kurtzman, and Donald L. Kreutzer. *Re-submitted.*

- **The Interleukin 1 Family of Cytokines and Receptors as Key Regulators of Human Breast Cancer Progression.**
  Alexander G. Pantschenko, Irina Pushkar, Kathleen H. Anderson, Yanping Wang, Lauri J. Miller, Scott H. Kurtzman George Barrows and Donald L. Kreutzer. *In preparation to be submitted.*

- **The Expression and Distribution of CXC (GRO and IL-8) Cytokines and Receptors (CXC-R2) in Human Breast Cancer.**
  Alexander G. Pantschenko, Lauri J. Miller, Kateri Fisher, and Donald L. Kreutzer. *Manuscript in preparation.*

2. ABSTRACTS.

- **Interleukin–1 Regulation of CXC Cytokine Expression in Human Breast Cancer (HBC) Tumor Cells.** Era of Hope 2002 Department of Defense Breast Cancer Research Program Meeting

3. FUNDING APPLIED FOR.

- University of Connecticut Health Center Research Advisory Counsel (HCRAC). 11/01. **"Breast Ductal Lavage: A Novel Diagnostic and Research Approach For Detecting and Characterizing Normal and Abnormal Ductal Epithelial Cells From Women at High-Risk For Breast Cancer"**
- Department of Defense Breast Cancer Idea Award. 6/01. **"Cooperation Among Tumor Cell Subpopulations That Express The CXC Family Of Cytokines And Receptors: The Role Of IL8 And GRO In Human Breast Cancer Angiogenesis And Tumor Progression."**

CONCLUSION.

Our present studies support the hypothesis that IL-1 and CXC cytokines and receptors form a network of effector pathways that are central to human breast cancer tumor progression. These effector networks comprise two main pathways: 1) the autocrine interactions/activation of tumor cell subpopulations (*e.g.* proliferation, migration, invasion etc.), as well as 2) the activation of VECs in a paracrine pathway resulting in tumor angiogenesis. Our present studies clearly demonstrate HBC tumor cell subpopulations *in vitro* and *in vivo*, and represents the foundation for our investigation on the role of IL-1 and CXC cytokine families in controlling HBC tumor progression. Although we have examined the expression (*in vivo* and *in vitro*) and activation (*in vitro*) of the IL-1 and CXC families of cytokines and receptors, we must directly demonstrate the role of these cytokine networks and tumor cell subpopulations in the pathogenesis of HBC. To directly determine whether tumor cell associated IL-1, IL-8, and GRO cytokines and/or IL-1 and CXC receptors are important for HBC *in vivo* tumor progression, we propose to future studies that utilize standard molecular techniques to regulate cytokines and receptor(s) expression in HBC tumor cell lines (*i.e.* suppress/delete or overexpress). Using molecular modifications of parental HBC cell lines, we will produce HBC cell subpopulations representing those tumor cell subpopulations we observe within the HBC tumor microenvironment. Utilizing these modified cells in comparison to the un-modified (parental) cells will allow us to clearly determine the importance of IL-1 and CXC cytokines and receptors in HBC tumor progression.

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APPENDIX

STATEMENT OF WORK (VERBATIM)

Proposal Title: The Role of Growth-Regulated Oncogene (GRO) Proteins in Human Breast Cancer Growth.

TASK 1. Evaluate GRO and GRO-Receptor (CXC-R2) Expression in Fresh and Archival Human Breast Cancer Patient Tumor Specimens.

Experimental Approach: (months 1-12).

1) All HBC tumor specimens are coded and evaluated as a double-blind study.
2) HBC tumor tissues are stained with anti-GRO and anti-CXC-R2
3) HBC tumor tissues are stained with anti-VWF and anti-CD31 to quantitate microvessel density.
4) HBC tumor tissue homogenates are used to quantitate GRO and CXC-R2 by ELISA, Western blot, and Quantative PCR.
5) Clinical and laboratory data for patient specimens including; tumor stage, estrogen-receptor, and progesterone-receptor expression are compiled.
6) For archival specimens, invasive, ductal carcinoma *in situ* (DCIS) as well as benign breast disease tissue (fibrocystic) specimens are available from our previous studies and from archival specimens in the Department of Pathology, University of Connecticut School of Medicine. Clinical outcome is factored as a variable and the data compiled.
7) Data are segregated based on tumor stage and statistical analysis services are available at the University of Connecticut School of Medicine.

TASK 2. Evaluate GRO/CXC-R2 expression (constitutive and inducible) in human breast cell lines *in vitro.*

Experimental Approach: (months 2-10)

1) Established human breast cell lines (see Table 1) are evaluated for GRO/CXC-R2 expression under unstimulated (baseline) and stimulated (IL1 and TNF) culture conditions.
2) Cell culture supernatant and cell lysates are assayed for GRO/CXC-R2 by ELISA, Western Blot, and Quantitative PCR.
3) HBC cell lines (as above) are cultured onto glass chamber slides and evaluated for GRO/CXC-R2 expression by immunocytochemistry.

TASK 3. HBC cell line proliferative responsiveness to GRO and GRO inducers (IL-1 and TNF) are assayed *in vitro.*

Experimental Approach: (months 12-24)

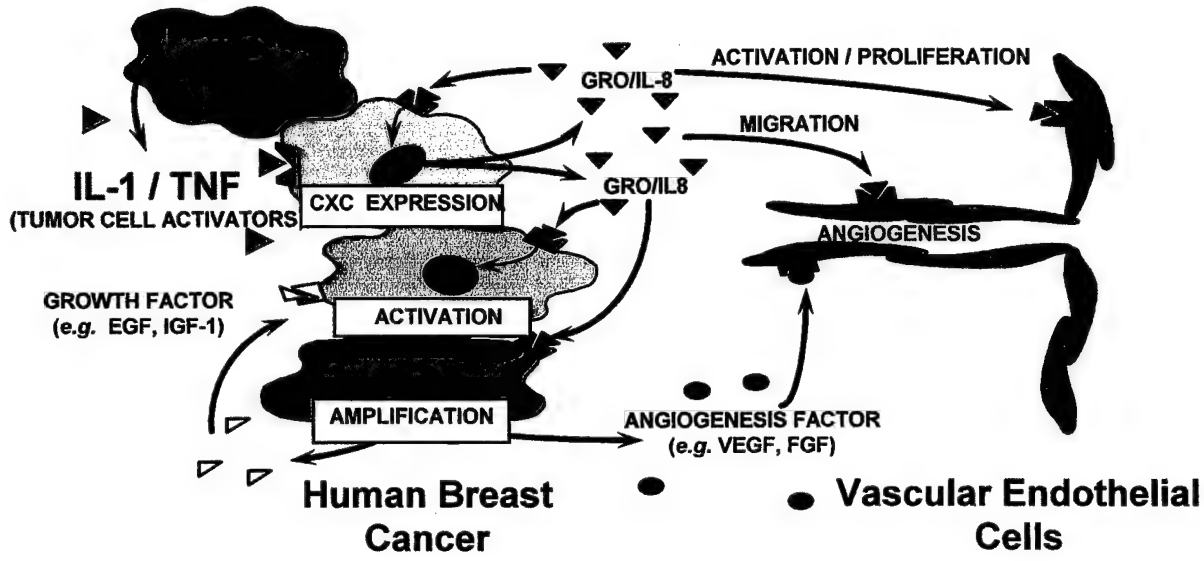
1) Established human breast cell lines are cultured with GRO, IL8, TNF, or IL1 and proliferation is measured by ^{3}H-thymidine incorporation. Proliferation responsiveness to cytokines is confirmed by determining specificity. Confirmation of specific response is determined with the addition of blocking antibodies for ligand and/or receptor, or antagonists, or antisense oligonucelotides [unmodified phosphodiester oligonucleotides complimentary to GRO, CXC-R2, or nonspecific (negative control)].

TASK 4. To determine the relative contribution of GRO/CXC-R2 as a tumor growth factor compared to GRO as an angiogenesis factor using the xenograft nude mouse model.

Experimental Approach: (month: 24-36)

1) HBC cells lines characterized for varying levels of GRO and CXC-R2 expressors as described in Task 2 & 3 are implanted into the nude mouse mammary gland fat pad such that high and low ligand and/or receptor cell lines are compared. The resulting tumors (or lack of establishing tumors) are evaluated as follows:
    a) Tumor Growth
        - Rate
        - Size
        - Histology, (hematixylin and eosin stain)
    b) Cytokine Expression
        - Immunohistochemistry for GRO, CXC-R2, IL1 and TNF
        - Q-PCR, ELISA, Western blot to quantitate GRO, CXC-R2, IL1 and TNF
    c) Angiogesesis
        - Immunohistochemistry to determine microvessel density using anti-VWF and anti-CD31

Figure 1.
Molecular Model of Human Breast Cancer Progression and the Role of IL-1 and CXC Cytokines Based on our Hypothesis.



Model Based on Hypothesis. Using our data and hypothesis, we have constructed a molecular model that describes tumor cell subpopulation cooperativity and the role of IL-1 and CXC (GRO & IL-8) families of cytokines and receptors in human breast cancer progression. In this model, tumor cell activators (such as IL-1 and TNF) induce CXC expression in HBC cells and the resulting CXCs, acting through CXC-Rs on the tumor cells, stimulate proliferation by autocrine action. Also, CXCs produced by breast tumor cells can bind to CXC-R2 expressed on vascular endothelial cells and support their activation, proliferation and migration leading to angiogenic support for tumor growth. Additionally, CXCs can not only mediated vascular endothelial cell migration, but is also an important tumor cell chemotactic factor. Furthermore, CXCs can induce a cascade of cytokine expression including secondary tumor growth factors [e.g. Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF)] as well as secondary angiogenesis factors [e.g. Vascular Endothelial Growth Factor (VEGF), or Fibroblast Growth Factor (FGF)].

Figure 2.

GRO and IL-8 Levels in Human Breast Cancer Tumor Tissue Homogenates.

Human breast cancer surgical samples collected at St. Francis Hospital and Medical Center, Hartford CT were homogenized and assayed by ELISA for cytokine/cytokine receptor levels and RIA for Estrogen receptor/Progesterone receptor (ER/PR) expression.

Cytokine / Receptor	Number of Samples	Mean ± sem (pg/mg TP)[a]	Range[b] (pg/mg TP)[a]
GROα [c]	100	9.14 ± 1.22	6.8 – 11.5
GROTOTAL [d]	98	1.64 ± 0.5	0.7 – 2.6
IL-8	103	106.4 ± 38.7	45.4 – 165.3
IL-1α	82	17.6 ± 3.9	10.0 – 25.5
IL-1β	101	12.0 ± 1.4	9.2 – 14.9
IL-1ra	65	18,741 ± 2,768	13,200 – 24,269
IL-1R1	70	15.3 ± 4.1	7.0 – 23.7
IL-1 RII	70	58.3 ± 8.2	41.8 – 74.8
ER	223	89.4 ± 6.5	76.6 – 102.2
PR	224	114.2 ± 10.8	93 – 135.4

a = Tumor homogenate cytokine levels are expressed as picograms per milligram of total protein and are represented as mean ± standard error of the mean.
b = Range is 95% confidence interval.
c = GROalpha measured using R&D reagents.
d = GROTOTAL assay recognizes GROalpha, GRObeta, and GROgamma using PharMingen reagents.

Figure 3.

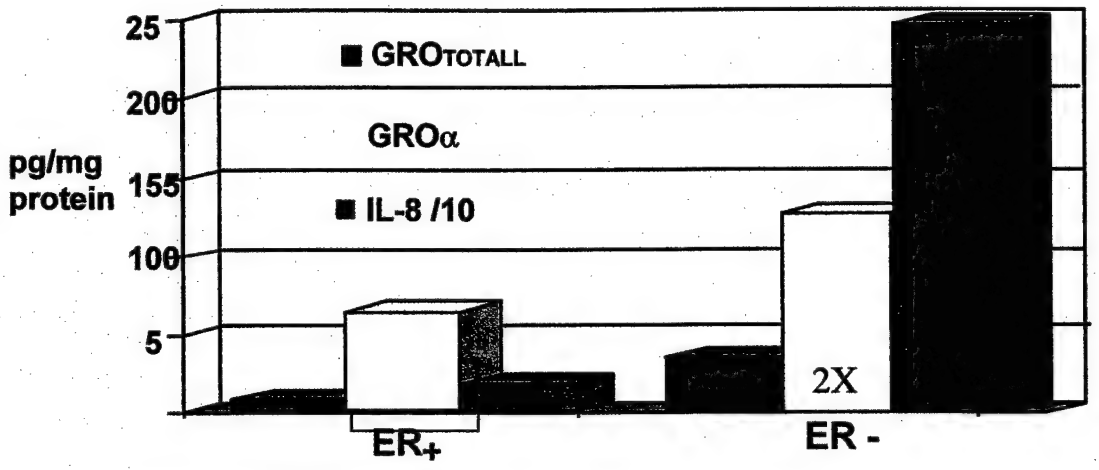
IL-8 and GRO Correlations in Human Breast Cancer Tumor Tissue Homogenates.

Comparison of Correlation for cytokine levels by linear regression analysis and Pearson correlation test. $p \leq 0.05$ is significant. Negative (-) value represents an inverse correlation.

	IL-8	IL-1α	IL-1β	IL-1ra	IL-1R1	IL-1R2	TNF-R1	TNF-R2	ER	PR
GROα p< (n)	*n.s. (58)*	*n.d.*	*n.d.*	*n. d*	*n. d*	*n. d*	*n. d*	*n. d*	**- 0.04 (99)**	*- 0.07 (97)*
GROTOTAL p< (n)	**0.00** (97)	**0.001** (30)	*n.s.*	*n.s.*	**0.001** (30)	*n.s.*	*0.09 (26)*	*n.s.*	**- 0.05 (98)**	*n.s.*
IL-8 p< (n)	**0.00 (170)**	**0.01** (82)	**0.01** (101)	*n.s.*	*n.s.*	*0.06 (70)*	*n.s.*	*n.s.*	**- 0.00** (170)	**- 0.02** (169)

n.s. **= not significant**
n.d. **= not determined**
(n) equals the number of samples analyzed
GROα measured using R&D reagents
GROTOTAL assay recognizes GROalpha, GRObeta, and GROgamma using PharMingen reagents

Figure 4.
GRO Expression Follows IL-8 Expression and Both Inversely Correlated To Estrogen Receptor (ER) Expression In HBC Tissue Homogenates.



GROα measured using R&D reagents
GROTOTAL assa recognizes GROalpha, GRObeta, and GROgamma using PharMingen reagents
3X, 2X, and 3X are fold difference between ER+ and ER – for GROTOTAL, GROα and IL-8 respectively.

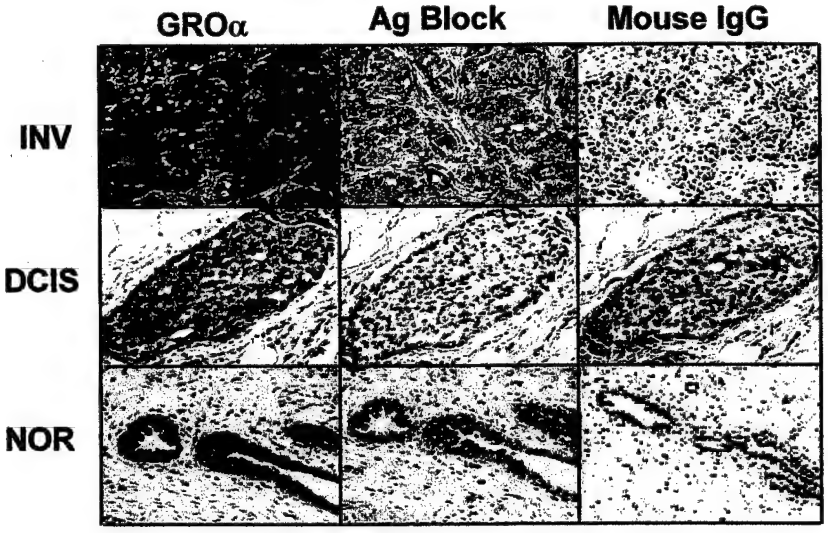
Figure 5.
GRO and IL-8 Levels are Significantly Different in ER+/ER- HBC Homogenates.

	ER+ pg/mg TP ± sem	*p*< ER + *vs.* ER –	ER – pg/mg TP ± sem
GRO$_{TOTAL}$ (number of samples)	0.93 ± 0.2 (61)	*p*< 0.02	2.81 ± 1.2 (37)
GROα (number of samples)	6.56 ± 0.6 (58)	*p*< 0.04	12.9 ± 2.7 (41)
IL-8 (number of samples)	49.4 ± 17.0 (110)	*p*< 0.00	206 ± 78.1 (61)

ER = Estrogen Receptor, TP = total protein
p value determine by log transformation of pg/mg TP and mean comparison by *t* -test.
GROα measured using R&D reagents
GRO$_{TOTAL}$ assay recognizes GROalpha, GRObeta, and GROgamma using PharMingen reagents

Figure 6.
Immunohistochemical Demonstration of GROα Expression in Breast Cancer Patient Tissue.

Tissue samples were assayed for GRO expression using anti-human GROα (R&D Systems). Specificity of the reaction was determined by using negative controls consisted of *1.* Ag Block (GROα is pre-incubated at a 100 molar excess with anti-human GROα antibody) and *2.* Mouse IgG (non-immune mouse serum is used as the primary antibody).

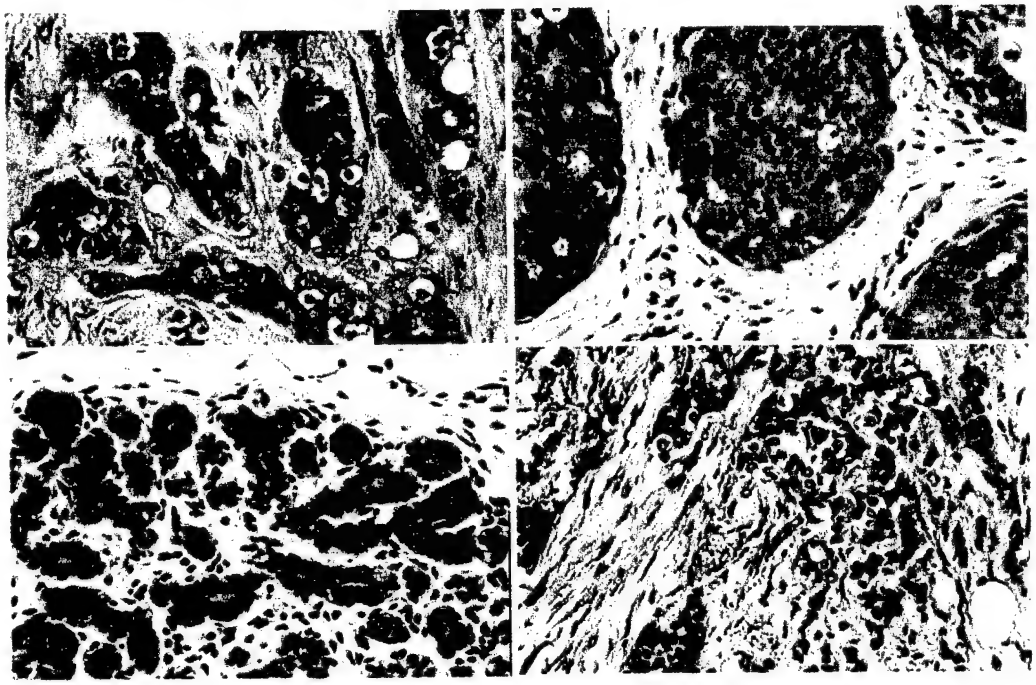


INV = Invasive (18 samples)
DCIS= Ductal Carcinoma *In Situ* (7 samples)
NOR = Normal (Mammary Reduction) (6 samples)

Figure 7.
Immunohistochemical Demonstration of
IL-8 Expression in Breast Cancer Patient Tissue.

Invasive anti-human IL-8

DCIS anti-human IL-8

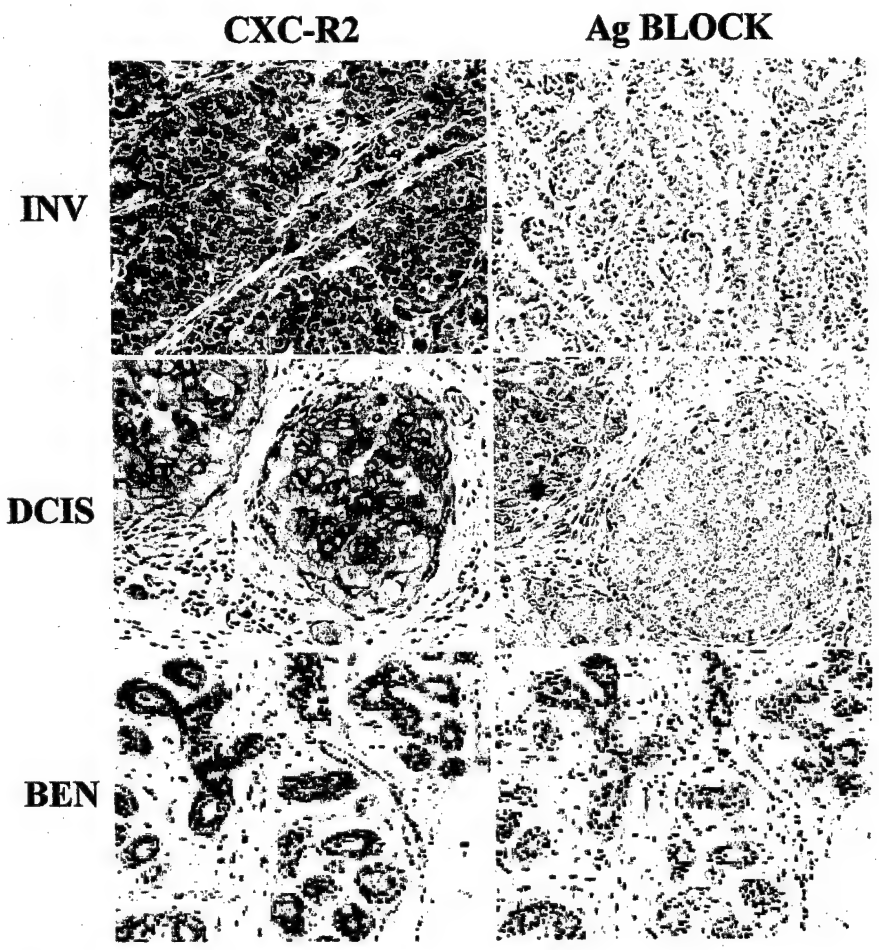


Fibrocystic anti-human IL-8

Pre-Immune mouse serum as negative control

DCIS= Ductal Carcinoma *In Situ*

Figure 8.
Immunohistochemical Demonstration of GRO Receptor (CXC-R2) Expression in Human Breast Cancer Patient Tissue.



CXC- R2 = anti-human CXC-R2
Ag Block = (CXC-R2 is pre-incubated at a 100 molar excess with anti-human CXC-R2 antibody)
INV = Invasive
DCIS = Ductal Carcinoma *In Situ*
BEN = Benign (Fibrocystic)

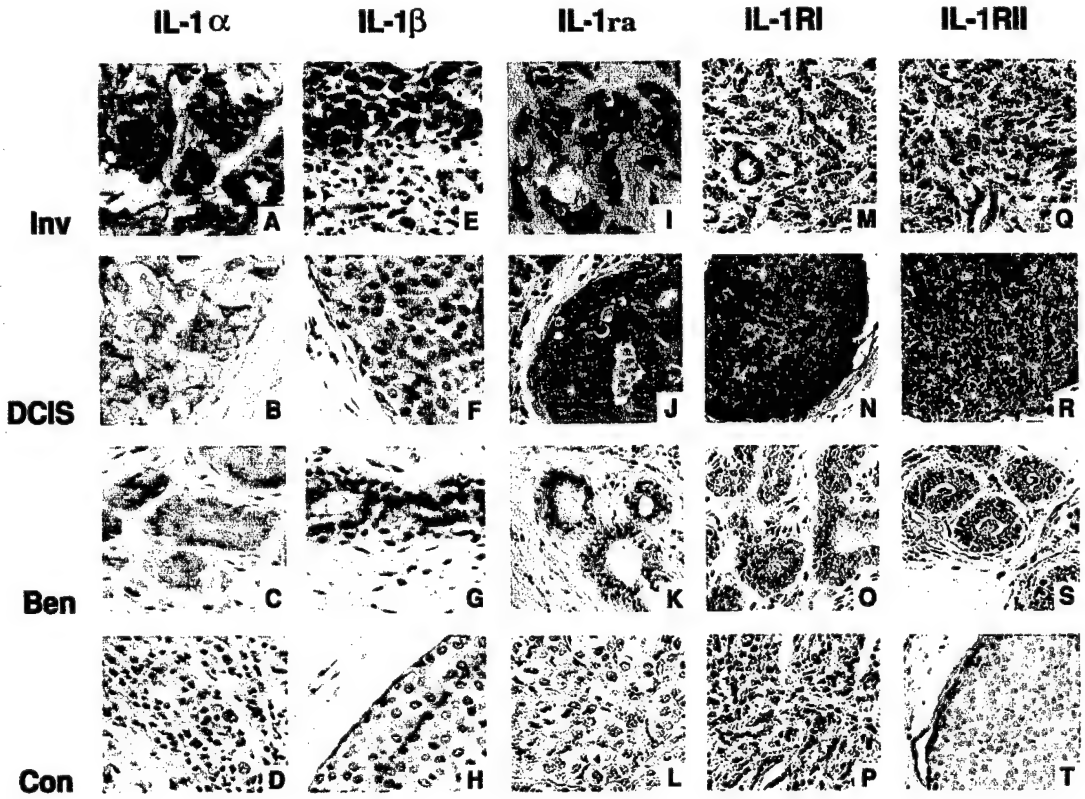
Figure 9.

Summary of CXC-L (IL-8 and GROα) CXC-R Expression in HBC Tissue: Percent of Positive Samples.

Tissue	IL-8	GROα	CXC-R1	CXC-R2
MALIGNANT				
Tumor Cells	+++	+++	+++	+++
SVECs	+++	---	++	+++
LVECs	+++	---	---	++
BENIGN				
Duct Epithelial	+++	+++	+	+
SVECs	+++	---	++	+++
LVECs	---	---	---	---

Immunohistochemical Scoring:
+++ > 90% of Specimens Positive
++ 51-90% of Specimens Positive
+ 21-50% of Specimens Positive
--- < 20% of Specimens Positive

Figure 10.
Immunohistichemical Demonstration of the IL-1 Family of Cytokines and Receptors in Human Breast Cancer Tissue.



Inv = Invasive
DCIS = Ductal Carcinoma *In Situ*
Ben = Benign (Fibrocystic)
Con = Control for immunoassay using species specific primary antibody

Figure 11.

Constitutive and Inducible *In Vitro* Expression of IL-8 and GROTOTAL By Human Breast Cells.

Human mammary epithelial and tumor cell lines were plated at 10^6 cells/well/ml^{-1} in 12-well flat bottom plates and incubated overnight at 37^{0}C in saturated humidity. The following day, the media was aspirated and replaced with fresh culture media. Triplicate wells are then treated with 10ng/well/ ml^{-1} of either TNFα, TNFβ, IL-1α, IL-1β, media alone, or 5□g/well/ml^{-1} LPS. The cells were incubated for an additional 24hrs. Each value represents the mean and standard deviation for three independent experiments.

IL-8 AND GRO_{TOTAL} Levels in Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	MEDIA		TNFα		TNFβ	
	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)
NORMAL						
HMEC	2,300 ± 430	2,631 ± 543	3,010 ± 1240	1,516 ± 209	1,620 ± 280	1,856 ± 331
ESTROGEN-INDEPENDENT						
BT-20	240 ± 50	25 ± 0	290 ± 10	25.0 ± 0	260 ± 50	25 ± 0
MDA-MB-231	1,080 ± 360	58 ± 15	7,300 ± 2,750	125 ± 20	2,040 ± 790	44 ± 8
ESTROGEN DEPENDENT						
MCF-7	290 ± 80	46 ± 17	270 ± 50	53 ± 14	290 ± 80	14 ± 3
T-47-D	110 ± 10	71 ± 13	110 ± 10	62 ± 0	110 ± 10	62 ± 0
ZR-75-1	240 ± 40	20 ± 4	150 ± 30	14 ± 5	160 ± 30	15 ± 4

IL-8 AND GRO_{TOTAL} Levels in Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	LPS		IL-1α		IL-1β	
	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)
NORMAL						
HMEC	3,390 ± 840	3,987 ± 445	25,060 ± 7,730	8,367 ± 1,106	15,080 ± 3,590	9,459 ± 765
ESTROGEN-INDEPENDENT						
BT-20	250 ± 40	25 ± 0	279,570 ± 45,510	2,379 ± 1,331	306,520 ± 12,500	3,953 ± 1,694
MDA-MB-231	136,210 ± 62,090	1,013 ± 139	358,830 ± 52,350	1,057 ± 128	356,960 ± 61,100	909 ± 122
ESTROGEN DEPENDENT						
MCF-7	330 ± 110	19 ± 4	320 ± 80	28 ± 5	400 ± 130	48 ± 18
T-47-D	100 ± 10	62 ± 0	310 ± 40	2,900 ± 774	350 ± 50	2,167 ± 1,194
ZR-75-1	1,980 ± 430	162 ± 22	29,260 ± 4,210	653 ± 50	32,600 ± 4,080	622 ± 131

Figure 12.

IL-1 is a Major Inducer of IL-8 and GROTOTAL Expression in Human Breast Cancer Cells *In vitro*.

Stimulation index for induced expression of IL-8 and GRO□ by human mammary epithelial and tumor cell lines. Stimulation index (S.I.) = [unstimulated (media) / stimulated (cytokine)] for human mammary epithelial and tumor cell lines. This data clearly demonstrates that IL-1 is a potent inducer of both IL8 and GRO, in particular, for the estrogen-independent human breast cancer cells lines BT-20 and MDA-MB-231.

IL-8 AND GRO_{TOTAL} Stimulation Index for Cell Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	MEDIA		TNFα		TNFβ	
	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)
NORMAL						
HMEC	1	1	1	1	1	1
ESTROGEN-INDEPENDENT						
BT-20	1	1	1	1	1	1
MDA-MB-231	1	1	7	2	2	1
ESTROGEN DEPENDENT						
MCF-7	1	1	1	1	1	0
T-47-D	1	1	1	1	1	1
ZR-75-1	1	1	1	1	1	1

IL-8 AND GRO_{TOTAL} Stimulation Index for Cell Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	LPS		IL-1α		IL-1β	
	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)
NORMAL						
HMEC	1	2	11	3	7	4
ESTROGEN-INDEPENDENT						
BT-20	1	1	1,138	95	841	158
MDA-MB-231	127	17	333	18	332	16
ESTROGEN DEPENDENT						
MCF-7	1	0	1	1	1	1
T-47-D	1	1	3	40	3	30
ZR-75-1	8	8	120	32	134	31

Figure 13.
Western Blot Analysis Demonstrating the Expression of CXC-R2 in the MDA-MB-231 Human Breast Cancer Cell Line and in HBC Tissue Homogenates (H1 and H2).

10^6 MDA-231 cells were cultured per well in 12 well tissue culture treated plates for 24 hrs with 10% fetal calf serum. Media was aspirated and replaced with fresh media without serum (Media) or media without serum and 50ng IL-8 (IL-8), or 50ng TNFα (TNF), or 1mcg lipopolysaccharide (LPS), or 10ng phorbol 12-myristate acetate (PMA), or 100nM dexamethasone21 acetate (Dex). After an additional 48 hrs of culture, the supernatant is aspirated and cell lysate is obtained using M-Per reagent (Pierce). Lysate samples are resolved on a 12% PAGE and transferred onto PVDF. CXC-R2 is detected using and CXC-R2 antibody (Santa Cruz), biotinylated Rabbit anti-mouse, and ploy HRP (Endogen). The reaction is visualized using Immun-Star HRP Chemiluminescent Kit (BioRad).

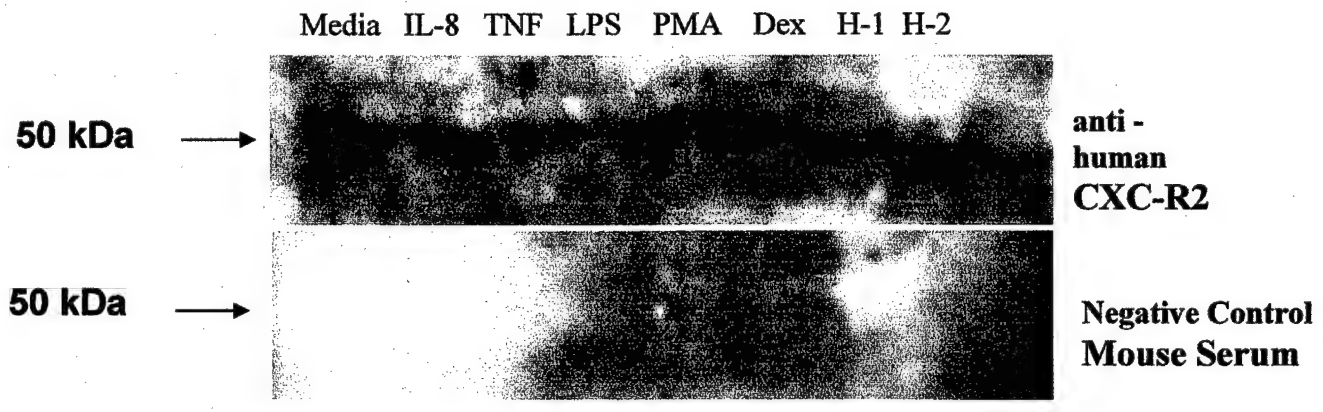


Figure 14.
Neither IL-8 or GROα Appear To Induce Proliferation in MDA-231 HBC Cells *In Vitro.*

$5x10^4$ MDA-231 cells/well/ml^{-1} were plated in 12 well culture plates in media with 2.5% fetal bovine serum and cultured for 24hrs. After the 24hr equilibration period the cells were then treated 100 µl of media with 0.2% BSA (Media) or 100 µl of treatment. Treatment consisted of a final concentration of 50ng/ml of IL-8 (IL-8), or 50ng/ml of GROα (GROα) 10% Fetal Bovine Serum (10% FBS). Cells were monitored daily for any obvious cytopathology. Cell count and viability were preformed by the trypan blue exclusion method.

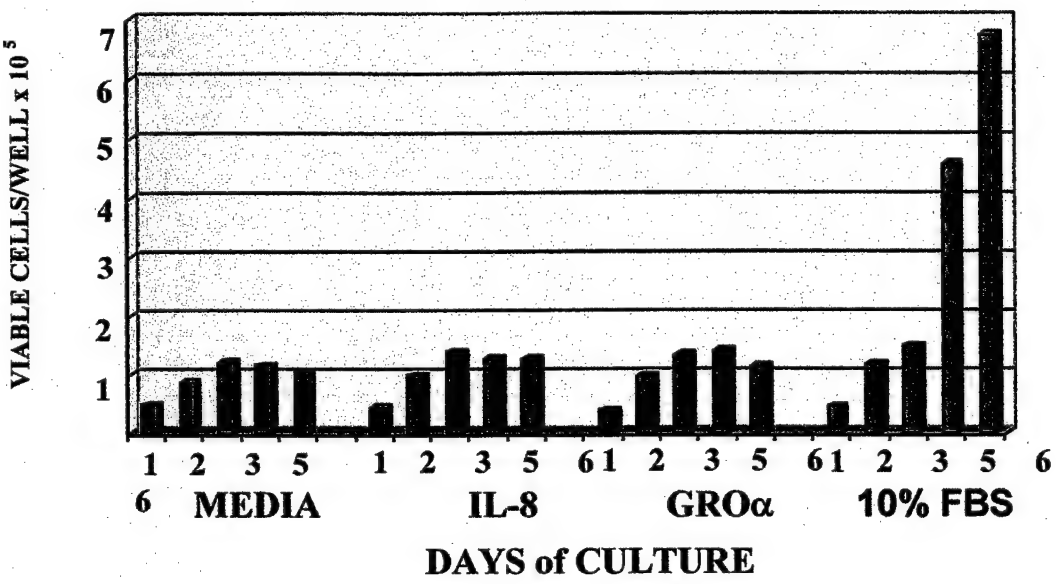


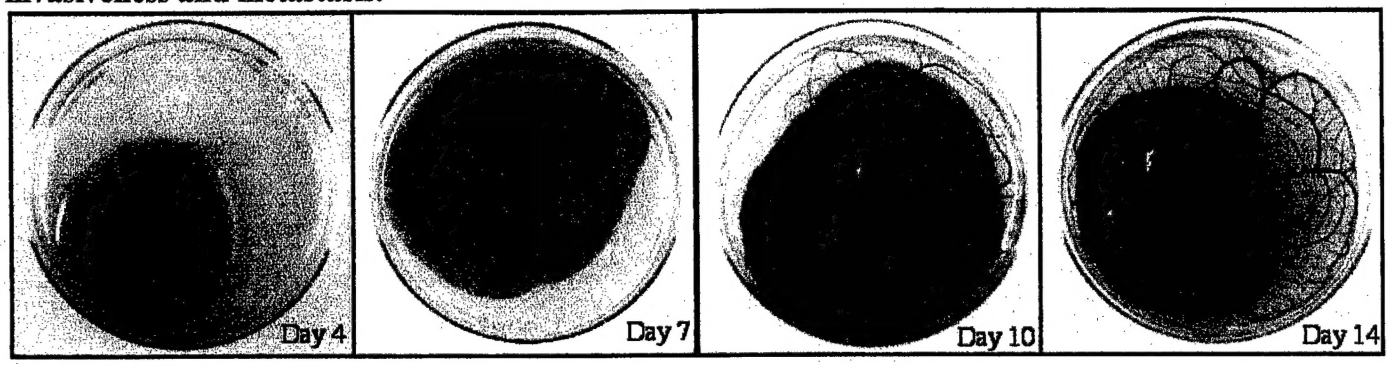
Figure 15.

Comparison Between the *Ex Ova* Chicken Embryo CAM Model and Mammalian Models for the Study of Tumor Progression. The CAM model possesses many advantages over mammalian models and thus justifies further studies to fully exploit this model for testing the role of tumor progression factors (*e.g.* cytokines and cytokine receptors).

Chicken Embryo *Ex Ova* CAM Models	Mammalian Models
Low Cost (eggs, time, labor, etc.) 1 Chick Embryo = $0.50	Moderate to High Cost (murine-canine, per diem. time, labor, etc.) 1 Nu Mouse = $32
Complete *In Vivo* Environment	Complete *In Vivo* Environment
Allows Direct Continuous Visualization of the Xenograft Site	Does Not Allows Direct Continuous Visualization of the Xenograft Site
Model Has Been Described for Use in Tumor Angiogenesis, Invasiveness, and Tumor Growth	Model Has Been Described for Use in Tumor Angiogenesis, Invasiveness, and Growth
Short Duration for Experimental Results (< 14 Days)	Xenograft Results can Take Up To Several Months
Tissue and Blood Samples are Easily Obtained	Tissue and Blood are Available in Larger Quantity But Require More Invasive Procedures
Embryos Do Not Need to be Restrained	Animals Often Need to be Restrained
Not Regulated by Animal Care Committee	Strict Regulation by Animal Care Committee
Cost Effective Model For Rare or Expensive Reagents (e.g. Cytokines, Antibodies)	Cost Ineffective Model For Rare or Expensive Reagents (e.g. Cytokines, Antibodies)
Easy to Target Xenograft Site with Reagents (e.g. Cytokines, Antibodies)	Difficult to Target Xenograft Site with Reagents (e.g. Cytokines, Antibodies)

Figure 16.
Chick Embryo Growth Kinetics for the *Ex Ova* CAM Model.

In the *ex ova* chick model the embryo is removed from the egg shell at day 3 and can be grown up to 21 days at which time the chick would be fully developed. These photographs demonstrate the rich vasculature associated with the chorioallantoic membrane that serves as a substrate for the implanted human breast cancer cells. This model can be used to study breast cancer cell angiogenesis, invasiveness and metastasis.



*** Photographs kindly provided by Ms. Uli Klueh**

Figure 17.
Phase Contrast Photomicrograph of Breast Cancer MDA-213 Cells Transfected w/ MG-3 Retrovector Expressing EGFP or Ds Red2.

Using fluorescently labeled breast cancer cells we can better understand the interaction between GRO/IL-8 and the CXC-Receptors in tumor progression. The labeled cells represent a known phenotype (e.g. GRO high expressor - GFP) used in comparison (vs. CXC-R Negative - DsRed2) within both the *in vitro* and *in vivo* models (see Figure 18).

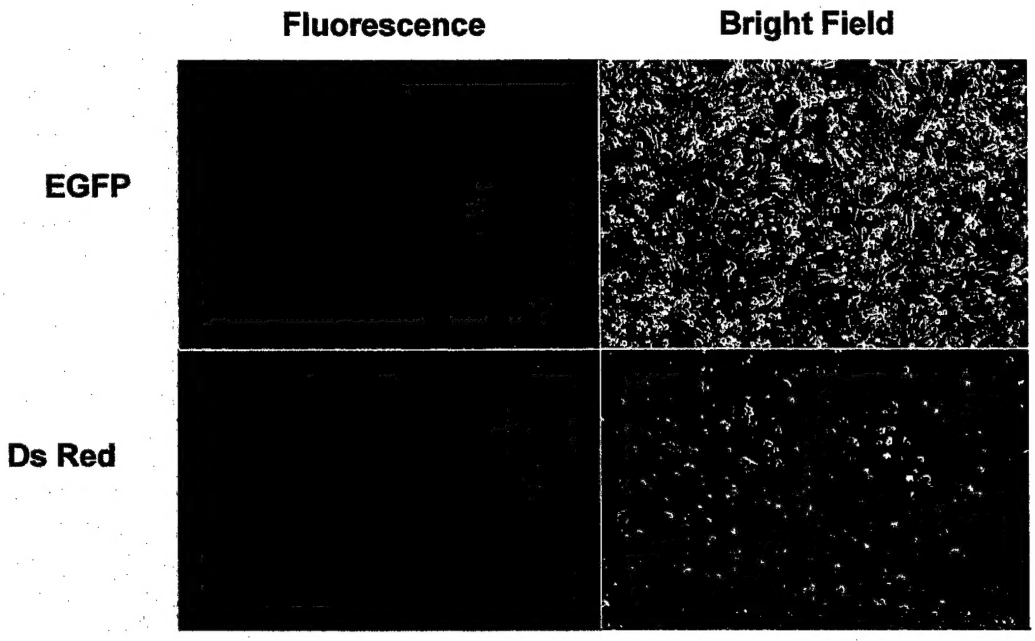


Figure 18.
Bright Field and Fluorescent Stereomicrograph of the Breast Cancer *Ex Ova* CAM Model.

In our preliminary studies we investigated the distribution and survival ability of tumor cells in the CAM. To begin to test the ability of tumor cells to grow in the *ex ova* CAM model, we used MDA-231 breast cancer cells transfected with a retrovector expressing the green fluorescent protein (GFP) gene. These studies demonstrate a simple method of tracking the presence and survival of cells on the CAM.

18 A+C; Bright field. Nylon mesh (N) is used to mark a test are on the CAM. The nylon screens have a 6mm hole punched in the center ("donut", Black Arrow) and the test tumor cells or tissue are placed in this open area. The nylon mesh "donut" is seen in the bright field photograph of the CAM, (Black Arrow).

18 B+D; Fluorescent. The same CAM nylon mesh is viewed under fluorescent light revealing significant fluorescent cell aggregates seen at 48 hours post-tumor cell placement (B), (White Arrow). As for the corresponding control, MDA- 231 non-GFP transfected tumor cells appear identical in the bright field, (C) but there is absolutely no detectable fluorescence signal, (D) thus showing specificity of MDA-231 GFP signal with no auto-fluorescent by the tumor cells, embryo or nylon mesh.

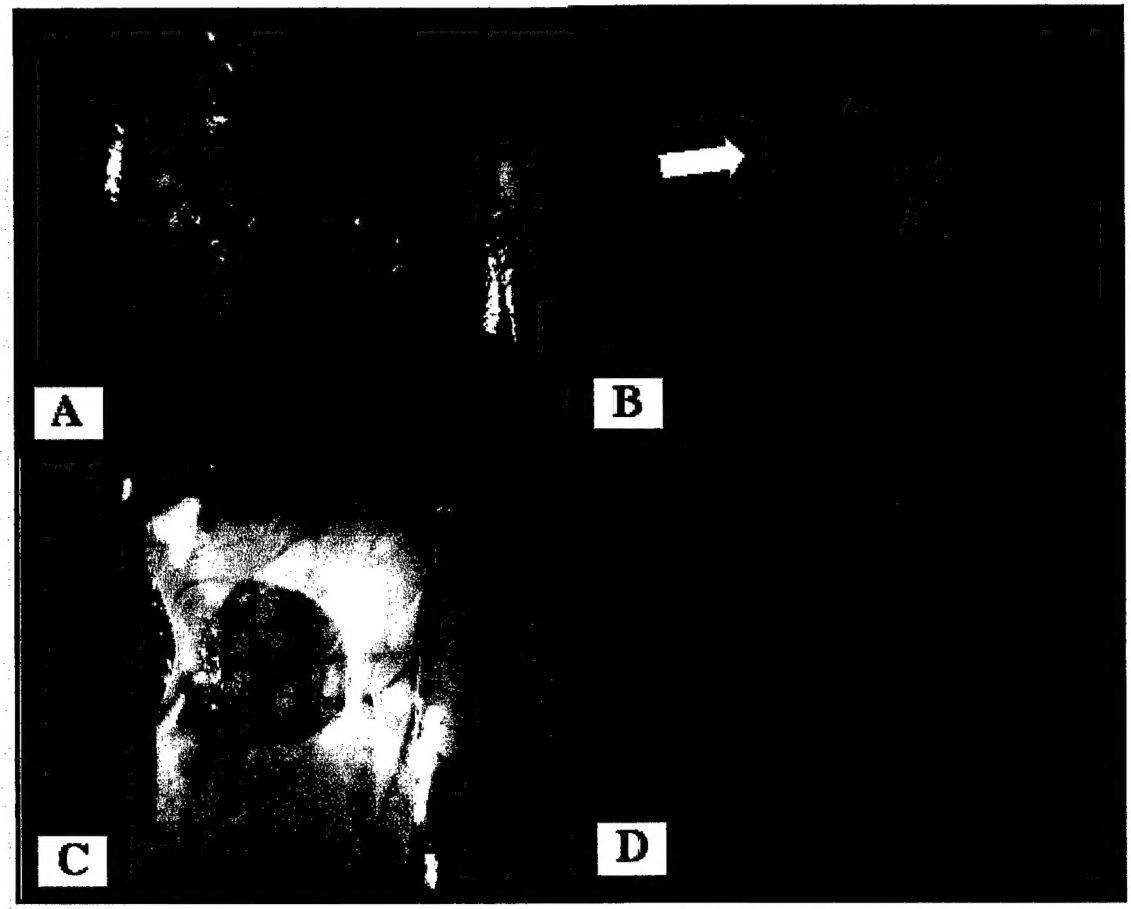


Figure 19.

Paraffin Embedded H&E Stained Normal CAM and Section of CAM with MDA-231 Cells.

Although the gross morphology suggests tumor cells are present in the CAM at 48 hours post placement (Figure 18). To directly demonstrate the presence and growth of the tumor cells, we histologically examined MDA-231 cells grown on the CAM. Control (no tumor cells) as well as CAMs with tumor cells, were fixed, processed, paraffin embedded, sectioned, and stained by H&E.

Panel A; The normal CAM shows a standard morphology with a thin layer of epithelial cells [outer chorionic epithelia (CE) and inner allantoic epithelia (AE)] blood cells, blood vessels (BV) and fibroblasts scattered throughout the stroma (S).

Panel B; The addition of nylon (N) mesh to the CAM did not induce significant inflammatory or other morphologic changes in the CAM structure.

Panel C+D; When tumor cells were added to the CAM and allowed to incubate for 48 hours significant tumor growth and invasion of the underlying CAM stroma by the advancing tumor mass (Black Arrow) can be observed. Thus, these studies indicate the feasibility of using the chorioallantoic membrane as a simple model to look at both tumor cell growth and invasion.